

**VERIFICATION OF TRANSLATION**

The undersigned hereby declares the following:

That I am knowledgeable in Japanese and English. That I have reviewed the Specification of JP 2004-056707 and verify that the attached document is an accurate translation thereof.

All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true. Further, these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

6 July 2009  
Date

Masahiro Abe  
Signature

## [DOCUMENT] Claims

1. A phosphoramidite method for the synthesis of a nucleic acid oligomer with the use of an alcohol-type compound as an activator.
- 5 2. A phosphoramidite method for the synthesis of a nucleic acid oligomer with the use of a mixture of an alcohol-type compound and an acid catalyst as an activator.
3. A method according to Claim 1 or 2, wherein the alcohol-type compound is selected from the group consisting of
- 10 hydroxybenzotriazole-1-ol (HOBt), a HOBt-derivative and a phenol analogue.
4. A method according to Claim 1 or 2, wherein the HOBt-derivative has substituents at its 4 and/or 6 positions.
5. A method according to Claim 4, wherein the HOBt-derivative
- 15 is 6-trifluoromethylbenzotriazole-1-ol,  
6-nitrobenzotriazole-1-ol, or 4-nitro-6-trifluoromethyl  
benzotriazole-1-ol.
6. A method according to Claim 3, wherein the phenol analogue  
is selected from the group consisting of 2,4-dinitrophenol,
- 20 3,4-dicyanophenol and 2-nitro-4-trifluoromethylphenol.
7. A method according to any one of Claims 2 ~ 6, wherein the  
acid catalyst is selected from the group consisting of  
imidazole, tetrazole and their derivatives.
8. A method according to Claim 7, wherein the acid catalyst is
- 25 benzimidazoletriflate (BIT), 4-ethylthiotetrazole,  
imidazolium triflate or 4,5-dicyanoimidazole.

9. A method according to any one of Claims 1-8, wherein a mixture comprising an equal amount of the alcohol-type compound and the acid catalyst is used as the activator.
10. A method according to any one of Claims 1-9 with the use  
5 of a solid phase support.
11. A nucleic acid oligomer prepared by the method according to any one of Claims 1-10, which is at least 10-mer.
12. A nucleic acid oligomer according to Claim 11, which is a DNA oligomer of 20-mer.
- 10 13. A nucleic acid oligomer according to Claim 11 or 12 to be used for a DNA chip

[DOCUMENT] Specification

[Title of Invention]

NOVEL METHOD FOR THE SYNTHESIS OF NUCLEIC ACID WITHOUT  
PROTECTING BASE MOIETY

5

[FIELD OF THE INVENTION]

[0001]

The invention relates to a method for the synthesis of a nucleic acid without protecting a base moiety, especially to a 10 phosphoramidite method for the synthesis of a nucleic acid oligomer using an alcohol-type compound as an activator.

[BACKGROUND GROUND OF INVENTION]

[0002]

15 H-phosphonate method wherein hydroxyl group-selective condensation is carried out by means of a phosphonium-type condensing agent, BOMP, has been known for the method of synthesizing DNA without protecting a base moiety (Non-Patent Document 1). This reaction utilizes the phenomenon that an 20 active phosphite intermediate generated during the condensation reaction will react more preferentially with a hydroxyl group than with an amino group in the base moiety.

[0003]

[Non-Patent Document 1] Wada, T.; Sato, Y.; Honda, F.; Kawahara, 25 S.; Sekine, M., Journal of the American Chemical Society 1997, 119, 12710-12821

[Non-Patent Document 2] Gryaznov, S. M.; Letsinger, R. L.,  
Journal of the American Chemical Society 1991, 113, 5876-5877

[SUMMARY OF THE INVENTION]

5 [Problems to be solved by the invention]

[0004]

However, in the above H-phosphonate method, the occurrence of such a side reaction of the base moiety as intramolecular cyclization will be exponentially increased in the DNA

10 synthesis as the length of a chain becomes longer. As a result, it will be very difficult to prepare a desired DNA oligomer as a main component after the synthesis has proceeded to give a 12-mer. Furthermore, DNA containing a cytosine residue would cause lots of the side reaction. There has been known no  
15 effective way to prevent said side reaction.

[Means for solving the problems]

[0005]

The present inventors have tried to solve the above problems  
20 by forming an active phosphite intermediate in the phosphoramidite method wherein a long chain oligomer can be easily synthesized, leading to a new method for the synthesis of DNA using hydroxyl group-selective phosphorylation.

[0006]

25 Thus, the present invention relates to a phosphoramidite method for the synthesis of a nucleic acid oligomer with the use of

an alcohol-type activator, preferably of a mixture or combination of the alcohol-type activator and an acid catalyst.

[Advantages of the invention]

5 [0007]

Up to now, the 12-mer was the longest oligomer that could be synthesized in the conventional methods without protecting the base moiety. According to the present invention, however, it is now possible to synthesize a DNA oligomer consisting of a 10-mer or a longer one, such as, for example, that consisting of a 20-mer with an extremely high purity on a solid phase. The resulting DNA oligomers may be advantageously used for a DNA chip.

15 [Best Mode for Carrying out the Invention]

[0008]

Any alcohol-type activator known to those skilled in the art may be used, being preferably selected from the group consisting of hydroxybenzotriazole-1-ol (HO<sub>B</sub>t), a HO<sub>B</sub>t-derivative and a phenol analogue in order to attain a high condensation efficiency (for example, 95 % or more). The HO<sub>B</sub>t-derivative preferably has 1-4 substituents such as nitro-, bromo-, iodo-, and trifluoromethyl group, being, for example, 6-trifluoromethylbenzotriazole-1-ol, 25 6-nitrobenzotriazole-1-ol, or 4-nitro-6-trifluoromethyl benzotriazole-1-ol. It is more preferably that the

HOBt-derivative has different substituents such as the trifluoromethyl and nitro groups at its 4 and/or 6 positions.

[0009]

Any phenol analogue known to those skilled in the art may be  
5 used, being preferably 2,4-dinitrophenol, 3,4-dicyanophenol and 2-nitro-4-trifluoromethylphenol in order to attain the high condensation efficiency as well.

[0010]

Any acid catalyst known to those skilled in the art may be used,  
10 being preferably imidazole, tetrazole and their derivatives such as, for example, benzimidazoletriflate (BIT), 4-ethylthiotetrazole, imidazolium triflate(trifluoromethane sulfonate) and 4,5-dicyanoimidazole.

[0011]

15 A ratio of the compounds in the combination of the alcohol-type activator and the acid catalyst may be optionally selected by those skilled in the art depending on conditions such as the kinds of each compound and reaction solvent, being usually an equivalent ratio of 1:10 - 10:1.

20 [0012]

The synthesis method of the present invention may be carried out in any system such as liquid or solid phase, being preferably carried out on a solid phase support for an industrial production of the oligomers. Any solid phase support known to  
25 those skilled in the art may be used, including CPG or HCP.

[0013]

The nucleic acid according to the present invention may be DNA or RNA, which may comprise not only natural-occurring bases but also their various variants or analogues having cyclonucleoside structure in a sugar moiety or various substituents at their 5 2' and/or 4' positions. Their phosphoric acid moiety may have phosphorothionate or methylphosphonate structure.

[0014]

Various reaction conditions in the phosphoramidite method, which are not specifically described in the present 10 specification, may be optionally selected by those skilled in the art.

[Examples]

[0015]

15 The present invention will be explained more in detail in line with the examples, which should not be construed to impose any limitations on the scope of the present invention.

[0016]

Example 1: Solid phase synthesis of dimer  
20 Selectivity for the hydroxyl group (hydroxyl group-selectivity) in the present invention was examined by using HCP solid phase support having an end thymidine introduced thereon. Phosphorylation was carried out for one minute between 20 equivalents of amidite units comprising each base 25 (A, C, G, T) and 40 equivalents of various activators for said end hydroxyl group on the HCP solid phase support, followed by

oxidation with 0.1 M iodine solution (pyridine: water = 9:1) for 2 min at a room temperature. A DMTr group was then removed with 3% trichloroacetic acid-CH<sub>2</sub>Cl<sub>2</sub> solution for one minute at a room temperature, and a phosphoric acid-protecting group 5 (2-cyanoethyl group) was excised with ammonia for 12 hours at a room temperature.

When the conventional activator, IMT, was used, d[ApT] and d[CpT] were obtained with the hydroxyl group selectivity of 77% and 83%, respectively. On the other hand, when HOBt was used 10 as the activator, d[ApT] and d[CpT] were obtained with the hydroxyl group selectivity of 99.7% 99.9%, respectively. The hydroxyl group-selectivity was calculated from a ratio of the area of peaks of the desired compound and N-phosphate.

[0017]

15 Example 2: Solid phase synthesis of trimer

Various trimers were synthesized in order to confirm utility of HOBt in the synthesis of DNA without protecting the base moiety. The dimmers were synthesized according to the method of Example 1 using HOBt as the activator, further followed by condensation 20 to produce trimers. When IMT was used, the production of a considerable amount of side-products was observed in the synthesis of d[TpApT] and d[TpCpT]. The results are shown in TABLE 1. It also shows the results obtained by using NBT, an activator for a proton-block method, in CH<sub>3</sub>CN-NMP mixture 25 solvent system.

[0018]

[TABLE 1]

compd	polymer	final product	% ratio of the desired product
2-11a	T-HCP	Apt	77.0% IMT
2-11c	T-HCP	CpT	82.9% NBT
2-11g	T-HCP	GpT	>99.9% HOBt
2-4	d[Apt]-HCP	TpApt	90.5% HO <sup>n</sup> Bt
2-4	d[CpT]-HCP	TpCpt	9.7% HO <sup>n</sup> Bt
2-4	d[GpT]-HCP	TpGpt	>99.9% DNP

[0019]

Example 3: synthesis of a long chain-oligomer using a DNA synthesizer

d[CCCCCTTTCTCTCTCT] and [TTAAAAATTATTAATTATT] were synthesized by means of DNA/RNA Synthesizer 392 (Applied Biosystem Inc. (ABI)). The synthesis of the DNA oligomer was carried out using HCP solid phase support having an end thymidine introduced thereon (1 μmol, 28 μmol/g, succinyl linker) and a mixture of 0.2 M  $\text{HO}^{\text{tf}}\text{Bt}$  (6-trifluoromethylbenzotriazole-1-ol: the alcohol-type activator) and 0.2 M BIT (benzimidazol triflate: the acid catalyst) in  $\text{CH}_3\text{CN}$ -N-methyl-2-pyrrolidone (15:1, v/v) solvent. Each elongation cycle of the synthesis is shown in TABLE 2.

[0020]

15 [TABLE 2]

step	operation	reagent(s)	time, (min)
1	washing	$\text{CH}_3\text{CN}$	0.2
2	detritylation	3% $\text{Cl}_3\text{CCOOH}$ / $\text{CH}_2\text{Cl}_2$	1.5
n			
3	washing	$\text{CH}_3\text{CN}$	0.4
4	coupling	0.1M amidite + 0.2M $\text{HO}^{\text{tf}}\text{Bt}$ + 0.2M BIT in $\text{CH}_3\text{CN}$ -NMP (15:1, v/v)	1.0
5	washing	$\text{CH}_3\text{CN}$	0.2
6	coupling	0.1M amidite + 0.2M $\text{HO}^{\text{tf}}\text{Bt}$ + 0.2M BIT in $\text{CH}_3\text{CN}$ -NMP (15:1, v/v)	1.0
7	washing	$\text{CH}_3\text{CN}$	0.2
8	oxidation	0.1M $\text{I}_2$ in Py-H <sub>2</sub> O-THF (20:2:78, v/v/v)	0.5
9	washing	$\text{CH}_3\text{CN}$	0.4

[0021]

The DMTr group was then removed with 3 % trichloroacetic acid in CH<sub>2</sub>Cl<sub>2</sub> (2 mL) for one minute, and the solid phase support was washed with CH<sub>2</sub>Cl<sub>2</sub> (1 mL × 3) and CH<sub>3</sub>CN (1 mL × 3). Finally 5 the solid phase support was treated with conc. ammonia water (500 μL) to be excised to give a desired product.

d[CCCCCTTTCTCTCTCT], Mass (M+H) calcd 5868.23, found 5869.92; Enzyme Assay dC:T = 1.00:0.99, isolated yield 79%.  
10 [TTAAAAATTATTAAATTATT], Mass (M+Na) calcd 6130.31, found 6132.69; Enzyme Assay dA:T = 1.00:0.94, isolated yield 31%.

[INDUSTRIAL APPLICABILITY]

[0022]

15 A DNA fragment having such a length as 20-mer or so is needed for an Affimetrix-type DNA chip, which is widely used for gene diagnosis. The success of the synthesis of such long DNA without protecting the base moiety has therefore made a start 20 for a high throughput preparation of the DNA chip with a high cost performance, making a great impact on biotechnology. The present invention is the first synthesis method that can attain a practical level without protecting the base moiety. It is expected that the nucleic acid oligomers synthesized according to the present invention will be utilized in gene diagnosis such 25 as SNP analysis.

[DOCUMENT] Abstract

[Problems]

It is intended to provide a novel method of synthesizing a nucleic acid oligomer whereby at least 10-mer of nucleic  
5 molecule oligomer (for example, a 20-mer) can be synthesized at an extremely high purity by the solid phase method without protecting a nucleotide base, compared with the conventional method without nucleotide base protection allowing the synthesis of a 12-mer at the highest.

10

[Means for solution]

A method for the synthesis of a nucleic acid oligomer, characterized in that an alcohol-type activator or a combination of an alcohol-type activator with an acid catalyst  
15 is used in the phosphoramidite method.